## REMARKS

In response to the Examiner's objection to the drawings, Applicants note that the purportedly offending Replacement Sheet (attached hereto as **Exhibit A**) was clearly not properly scanned by the Office, thereby leaving the "Precipitate" portion of the legend, and the majority of the phase diagram, un-blackened. A comparison of the photocopy that is **Exhibit A** with a downloaded copy of that same Replacement Sheet from the Patent Application Information Retrieval (PAIR) System (attached hereto as **Exhibit B**) shows that even O.I.P.E.'s intake stamp in the upper left-hand corner did not properly scan. Applicant's submit herewith (not attached hereto) this *Response to Non-Final Rejection*, a more blackened copy of that Replacement Sheet for Figure 1 for the Office's proper re-scanning.

In response to the Examiner's objection, Claim 1 has been amended to clarify that the "salt," therein referred to, is a salt of the "organic amine," also therein referred to.

In response to the Examiner's rejection under 35 U.S.C. §112, Claim 2 has been amended to more definitely claim a method wherein two aqueous solutions are being admixed over an extended period of time.

All of the elected claims (1-7) stand rejected as being obvious under 35 U.S.C. § 103(a) over Andrianov *et al.*, [*Preparation of hydrogel microspheres by coacervation of aqueuos polyphosphazene solutions*, Biomaterials 19 (1998) 109-115](hereinafter "Andrianov") in view of Pelta *et al.*, (*DNA Aggregation Induced by Polyamines and Cobalthexamine*, The Journal of Biological Chemistry, Vol. 271, No. 10, Issue of March 8, pp. 5656-5662, 1996)(hereinafter

"Pelta"). Those rejections are traversed below.

## I. Coacervate Systems are not Microspheres

The instant claims are directed to a method of producing polyphosphazene microspheres by admixing aqueous solutions containing a water-soluble polyphosphazene and an organic amine, such as spermine.

The primary reference, Andrianov, teaches the production of coacervate micro**droplets** from aqueous solutions of polyphosphazenes upon addition of sodium chloride as a coacervating agent. Andrianov further teaches that these polyphosphazene micro**droplets** may be stabilized through polymer crosslinking by way of the addition of calcium chloride, to then yield polyphosphazene micro**spheres**. Significantly, the addition of sodium chloride does not lead to micro**spheres**, but rather only leads to the formation of coacervate systems.

Coacervates are unstable systems and they are not identical to micro**spheres**:

> "...Coacervates may be described as liquid crystals and mesaphases...
> Coacervate systems are in dynamic equilibrium and alteration of the conditions may result in either the reformation of a one phase system or the formation of a flocculate or precipitate [1]." (See Page 285 of D. J. Burgess "*Complex Coacervation: Microsphere Rormation*" in "Macromolecular Complexes in Chemistry and Biology," P. Dubin *et al.*, Eds., Springer-Verlag, Berlin, Heidelberg, 1994)(attached hereto as **Exhibit C**).

Coacervates are required to be *cross-linked* in order to form micro**spheres**:

> "Although many successful coacervate microencapsulation systems have been prepared coacervate microcapsules have a number of limitations:...**they require stabilization by use of cross-linking agents** or heat..." (See Page 293 of D. J. Burgess "*Complex Coacervation: Microsphere Rormation*" in "Macromolecular Complexes in Chemistry and Biology," P. Dubin *et al.*, Eds., Springer-Verlag, Berlin, Heidelberg, 1994)(attached hereto as **Exhibit C**) (Applicants' emphasis added).

The method described by Andrianov cannot be used to produce micro**spheres** without this additional step of *cross-linking* of coacervate systems with calcium salts or other ionic cross-linkers.

Andrianov makes no mention of *organic amines* in general, or of *spermine* in particular. In fact, on the use of poly[di(carboxylatophenoxy)phosphazene] for microencapsulation, Andrianov teaches away from the use of organic solvents altogether:

> "The ability of poly[di(carboxylatophenoxy)phosphazene], PCPP (1) to form an ionotropic gel in the presence of calcium ions under mild physiological conditions and **without need of organic solvents** or elevated temperatures makes it an attractive candidate as a material for microencapsulation [4-6]." (See Andrianov, page 109, last ¶, first column)(Applicants' emphasis added).

Accordingly, nothing in Andrianov would suggest that polyphosphaneze microspheres could be formed by using an organic amine as a coacervating agent (instead of NaCl). Much less does Andrianov suggest using an organic amine in a one-step process to produce polyphosphazene microspheres, instead of a two-step process involving sodium chloride followed by calcium chloride.

The secondary reference, Pelta, teaches the use of polyamines, such as spermine, to precipitate DNA molecules. Pelta makes no mention of *polyphosphazenes* or of *microspheres*. The Examiner's rejections are based on the assumption that the artisan would consider it obvious to substitute one coacervating agent, spermine, for another coacervating agent, sodium chloride, to thereby produce polyphosphazene micro**spheres**. This assumption is erroneous; nothing in Pelta indicates or suggests that his use of spermine to precipitate DNA ultimately results in the formation of *DNA* micro**spheres**, ergo much less could Pelta suggest that the use of spermine with a polyphosphazene would ultimately result in the formation of *polyphosphazene* micro**spheres**. In fact, Pelta itself suggests that the use of spermine, or his other organic amines,

with DNA results in nothing other than the (micro**droplet**) coacervate system recognized by the rebuttal art discussed above:

> "**We have seen above that such aggregates can be considered as coacervates.** It is useful to recall here Oparin's classic proposal on the role of coacervation in prebiotic chemistry (39): coacervation is considered as an essential concentrating process by which mixtures of randomly formed prebiotic polymers initially in dilute solutions are condensed into concentrated assemblies. The phase separation of the polymers into separate **coacervate droplets** is thought to provide the appropriate medium required for the evolution of these prebiotic systems. According to the current view, Oparin's proposal suffers from the defect that it considered polypeptides rather than nucleic acids as a model for the primeval gene. The proposal of Baeza and co-workers as well as our present results should help reactualize Oparin's coacervation model in the perspective of a DNA (or an RNA) world." (See Pelta page 5661)(Applicants' emphasis added).

Accordingly, *at the time the invention was made* the artisan having the guidance of Andrianov and Pelta, would **not** have *reasonably expected* that (1) the substitution of Andrianov's NaCl, for Pelta's spermine, *and* (2) the elimination of Andrianov's calcium chloride, would produce a polyphosphazene microsphere.

In fact, contrary to the Examiner's position, Pelta suggests a lack of interchangeability of NaCl and organic amines as coacervating agents, insofar as their co-presence in DNA solution *prevents* rather than expedites the precipitation of DNA:

> "**The presence of an increased NaCl concentration prevents DNA precipitation as observed previously for spermidine and spermine (12, 14).** In the case of spermidine no precipitation is observed for a NaCl concentration greater than 100 mM. This concentration does not fully prevent the precipitation by spermine or cobalthexamine. The suppressing effect can be observed at higher concentrations (500 mM for spermine, 1.2 M for cobalthexamine)." (See Pelta page 5657)(Applicants' emphasis added).

Succintly, in the present invention spermine is used to produce polyphosphazene microspheres. Neither of the cited references, whether considered alone or in combination, suggests that an organic amine can be used to produce polyphosphazene microspheres. At best, the primary reference teaches a two-step process for making polyphosphazene microspheres

(neither of which involves an organic amine), and the secondary reference teaches the precipitation of DNA with an organic amine. Such disclosures do not suggest that an organic amine can be used to produce polyphosphazene microspheres.

In view of the foregoing, Applicants submit that independent claim 1 is non-obvious over Andrianov in view of Pelta. The remaining claims, all of which depend from independent claim 1, are therefore similarly non-obvious over Andrianov in view of Pelta.

In further view of the foregoing, Applicants submit that the application is in condition for allowance, and they therefore request its prompt passage to issue.

It is believed that no further fees are due. However, if any further fee is due it should be charged to Deposit Account No.: 03-0678. Similarly, any credit for overpayment should be credited to Deposit Account No.: 03-0678.

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Replacement Sheet



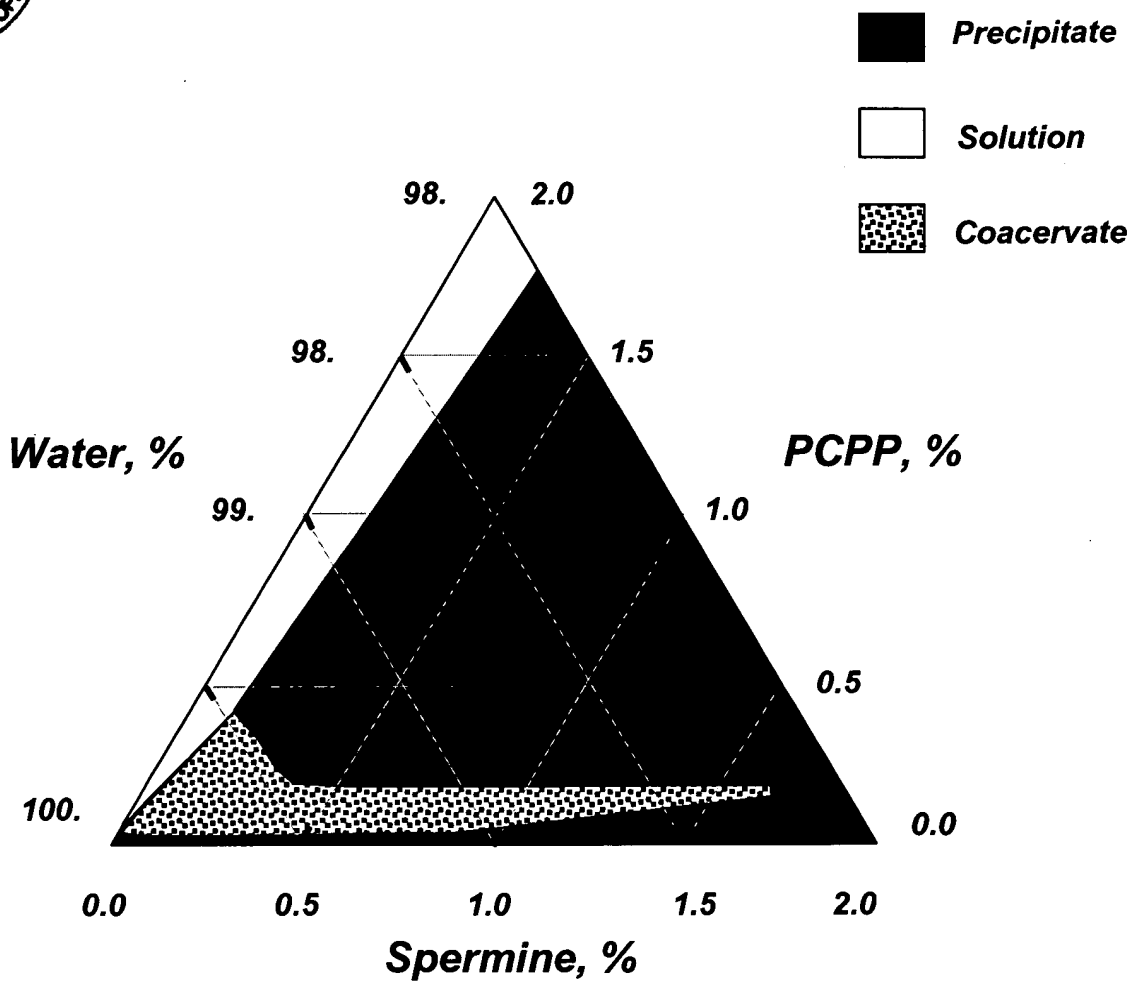


FIG. 1.

P. Dubin, J. Bock, R. Davis, D.N. Schulz, C. Thies (Eds.)

# Macromolecular Complexes in Chemistry and Biology

With 196 Figures and 39 Tables

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Hong Kong Barcelona Budapest

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ISBN 3-540-57166-3 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-57166-3 Springer-Verlag New York Berlin Heidelberg

Library of Congress Cataloging-in-Publication Data
Macromolecular complexes in chemistry and biology/P. Dubin . . . [et al.],
eds. p. cm.
Includes bibliographical references.
ISBN 0-387-57166-3
1. Polymer solutions. 2. Polyelectrolytes. 3. Complex compounds.
QD381.9.S65M32 1994 547.7'0454--dc20 93-38677 CIP



SPIN: 10076493 3020 5 4 3 2 1 0 Printed on acid-free paper

For EWP precipitation, larger molecular weight has higher efficiency in protein removal. However, in lysozyme precipitation, protein removal is independent of molecular weight except for *MW* 5000 PAA. It is likely that highly charged protein, which has strong electrostatic interactions with PAA, is less affected by the molecular weight of polyelectrolyte.

Particle size was affected by polyelectrolyte molecular weight. Larger molecular weight PAA gave larger precipitates probably as the result of a bridging effect.

For turbidimetric titrations in the binary system, ovalbumin lowers the critical pH of lysozyme less than 0.1 pH unit perhaps as the result of weak interference with PAA/lysozyme complexation. For turbidimetric titrations of single proteins and mixtures, larger molecular weight PAA gave higher critical pH values. In contrast, increased ionic strength lowers the critical pH value as a result of electrostatic screening.

*Acknowledgement.* This work was supported by National Science Foundation Grant No. ECE-8514865.

## 16.5 References

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# 17 Complex Coacervation: Microcapsule Formation

Diane J. Burgess

Simple and complex coacervation and the various theories of coacervation are reviewed. The Tainaka theory, which is the most broadly based of all the theories, was considered the most generally applicable. However this theory alone does not give a full explanation of the coacervation process.

The application of coacervation for the production of pharmaceutical microcapsules is discussed. There are a number of potential advantages in the microencapsulation of pharmaceutical and other products. Encapsulation by coacervation is an uncomplicated process which does not involve any elaborate manufacturing equipment. However this process usually involves the use of chemical crosslinking agents such as glutaraldehyde which have potential toxicity problems. In addition the application of these agents may be harmful to the encapsulated material. A complex coacervation process which is stable without the use of crosslinking agents or the application of heat is described. Complex coacervate microgels are formed between albumin and acacia under specific pH and ionic strength conditions. These microgel systems maintain stability under the following conditions: aging (six week study period); dilution with distilled water; change in pH (3.0 to 9.0); and change in ionic strength (1 to 80 mM).

## 17.1 Introduction and Terminology

Coacervation is the separation of a macromolecular solution into two immiscible liquid phases: a dense coacervate phase, which is relatively concentrated in the macromolecules, and a dilute equilibrium phase [1]. The word coacervate is derived from the Latin: *acervus*, meaning aggregate or heap, with the prefix, *co*, to signify the preceding union of the macromolecules. Coacervates may be described as liquid crystals and mesaphases. When only one macromolecule is present, this process is referred to as simple coacervation and when two or more macromolecules of opposite charge are present it is referred to as complex coacervation [1]. Simple coacervation is induced by a change in conditions which results in molecular dehydration of the macromolecules. This may be achieved by the addition of a non-solvent, the addition of microions or a temperature change. Complex coacervation is driven by electrostatic interaction between two or more macromolecules.

Coacervate systems are in dynamic equilibrium and alteration of the conditions may result in either the reformation of a one phase system or the formation of a flocculate or precipitate [1]. For example, the addition of excess non-solvent to an aqueous gelatin simple coacervate will result in precipitation. The phenomenon of coacervation is closely related to precipitation and flocculation.

In both simple and complex coacervation solvent is entrapped between the loops of the macromolecules as they associate. This is known as occlusion of solvent. Coacervates are more fluid compared to other systems with higher structural order, such as micelles. It is assumed that site specific interactions do not occur within the coacervate, therefore allowing freedom of movement of the individual molecules. Site specific interactions may lead to precipitation rather than coacervation if the charge densities of the molecules are sufficiently high.

## 17.2 Simple Coacervation

Simple coacervation may result on the addition of a dehydrating agent (coacervating agent) which promotes polymer-polymer interactions over polymer-solvent interactions. Bungenberg de Jong [1] has reported the induction of coacervation of aqueous gelation solutions on addition of sodium sulfate or ethanol. According to the Huggins theory of phase separation [2, 3], the interaction parameter ($X$) is increased on coacervation. $X$ is a dimensionless quantity proportional to the difference in interaction energy between a macromolecular segment-solvent contact and an equivalent number of solvent-solvent and macromolecule-macromolecule contacts. This parameter must be above a certain critical minimum value in order for coacervation to take place. $X$ may be increased by temperature reduction and therefore phase separation may result upon cooling [4]. It has been shown that the critical value of $X$ ($X_{cr}$) which is necessary for coacervation to take place is dependent on the molecular weight of the polymer [4]:

$$X_{cr} = 1/2(1 + r^{-1/2})^2$$

where $r$ is the ratio of molar volumes of solute to solvent, and therefore $r$ is proportional to the molecular weight of the solute. Higher molecular weight species will give smaller values of $X_{cr}$ and therefore coacervate more readily.

As $X$ is increased intramolecular associations result between the loops of the macromolecules causing a reduction in the volume over which the macromolecules extend. This can be measured by a decrease in the viscosity of the system. As more dehydrating agent is added intermolecular associations also increase, resulting in the production of a coacervate. The coacervating agent tends to be excluded from the coacervate phase, thus allowing more freedom of movement of the polymers within the coacervate. This is possible as the associations between the molecules are dynamic. The two phase coacervate system is in equilibrium and is readily reversible.

Simple coacervation is dependent on the structure of the macromolecules. Khalil et al. [5] and Nixon et al. [6] have shown the importance of the gelatin molecules being in a compact coil structure for simple coacervation to take place. Gelatin molecules attain this structure at pH values close to their isoelectric pH (pI) values and at low ionic strength. The compact coil configuration results from the intermolecular attractive forces which occur at the pI value due to the charges on the molecules being balanced at this pH. When the pH is altered away from the pI value the charges on the molecules are no longer balanced and the resulting repulsive forces cause unfolding of the molecules and this prevents coacervation from taking place. Similarly the addition of salt causes a decrease in the intramolecular attractive forces due to screening of the charged functional groups and results in molecular unfolding.

Therefore simple coacervation will not take place at salt concentrations above a critical maximum salt concentration [6]. Simple coacervation is suppressed at high macromolecule concentrations. Under conditions where the macromolecule concentration approaches that normally found in the coacervate phase there is little energetic advantage in the formation of a coacervate phase.



## 17.3 Complex Coacervation

Complex coacervate formation is dependent on a number of factors, including: pH, ionic strength, macromolecular weight, concentration and mixing ratio [1, 7]. Charge is a most significant factor for complex coacervation, and unless the two polyions carry opposite charges coacervation will not take place. The amount of charge carried by each polyion is also critical. At very low charge densities coacervation is suppressed [8–10] and at very high charge densities precipitation and/or gelation may occur [1, 9, 11–14]. The pH and ionic strength of the medium affects the charges carried by the polyions and therefore affects coacervation. Coacervate yield is optimized for an equal ratio mixture of macromolecules, by weight, at the pH value where they carry equal and opposite charges [7]. This pH is known as the electrical equivalence pH (EEP). Figure 17.1 shows a typical effect of change in pH on coacervate yield for a gelatin/acacia complex coacervate system (all other parameters are held constant at optimum values) [1, 7]. The mixing ratio of the macromolecules affects the extent of coacervation, since the electrostatic interaction will be altered depending on the amount of each macromolecule available to interact. Figure 17.2 shows a typical effect of macromolecule mixing ratio on coacervate yield for two macromolecules which carry equal charges (all other parameters are held constant at optimum values) [1, 7]. The optimum concentration ratio for maximum coacervate yield will vary depending on the ratio of the charges [7].

The molecular weight of the macromolecules must fall within a critical range for complex coacervation to take place [15–21]. High molecular weight materials will form gels or precipitates and low molecular weight materials will interact by ion pairing rather than coacervation [11, 12]. Coacervation is enhanced at low temperatures due to increase in solvent-solvent, solvent-solute and solute-solute interactions [22–25]. Coacervation is suppressed at high macromolecule concentrations [1, 7]. This effect is also observed for simple coacervation [1]. In highly concentrated systems, the concentration of the mixture will approach that normally found in the coacervate and as a result the

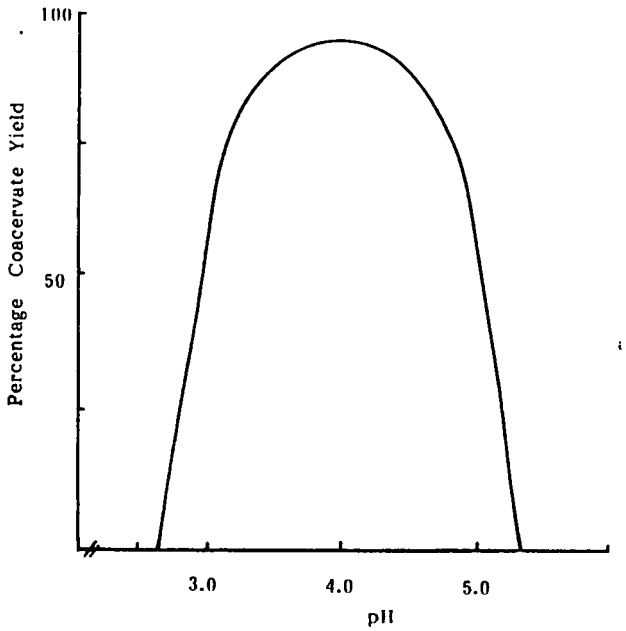


Fig. 17.1. The effect pH on coacervate yield for a typical gelatin/acacia coacervate system. (All other parameters held constant)

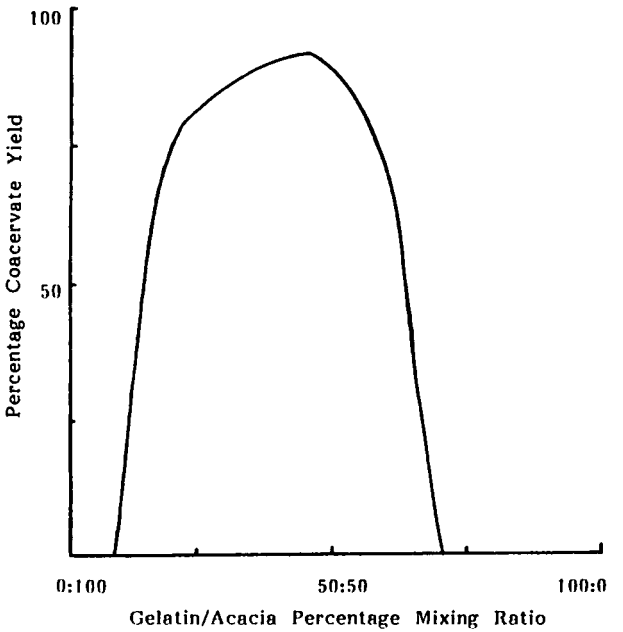


Fig. 17.2. The effect of macromolecule mixing ratio on coacervate yield for a typical gelatin/acacia complex coacervate system. (All other parameters held constant)



energy gain on formation of a coacervate is greatly reduced. In these concentrated mixtures the oppositely charged macromolecules will be in close proximity with one another and the molecular skeins may overlap. Thus there will be no advantage in forming a separate coacervate phase. Therefore, once a critical macromolecule concentration is reached coacervation will be completely suppressed. This phenomenon is termed 'self' suppression [1].

Suppression of coacervation at high salt concentration, known as 'salt' suppression [1], occurs as a result of the microions forming a dense atmosphere around the macromolecules and thus preventing electrostatic interaction. Coacervation may also be suppressed at low salt concentrations [8–10]. This effect is considered to be a result of the higher charge carried by the macromolecules under these conditions. Highly charged molecules will be in the extended molecular conformation which does not favor coacervation.

## 17.4 Theory of Complex Coacervation

There are four different theories of complex coacervation. The oldest is the Voorn–Overbeek theory [15–21], which was developed using the data of Bungenberg de Jong for gelatin/acacia complex coacervation [1]. The Voorn and Overbeek theory is based on the following assumptions: that there is a random chain distribution of macromolecules in both phases; that solvent-solute interactions are negligible; and that the interactive forces are distributive in nature with the system behaving as though the charges are free to move. If site specific interactions were to take place then precipitation rather than coacervation would be expected to occur. The first assumption implies that coacervation is limited to systems of relatively low charge density unless the ionic strength is high, as otherwise the macromolecules would be in the unfolded state rather than the random coil configuration. If the macromolecules are present in the extended state it would be difficult to entrap water within the molecular skeins and coacervation would be unlikely to occur. Voorn and Overbeek [15–21] interpreted coacervation as a competition between the electrostatic forces which tend to accumulate the charged macromolecules and entropy effects which tend to disperse them. They also regarded the solvent content of the coacervate in terms of its entropy contribution, as this allows a number of possible arrangements of the macromolecules. The electrostatic forces are assumed to be distributive in nature to account for the fluidity of the coacervate. According to the Voorn and Overbeek theory [15–21] the factors which affect complex coacervation are the charge densities and molecular weights of the macromolecules, the temperature, the dielectric constant and the Huggins interaction parameter (which was assumed to be negligible). Coacervation is considered to be a spontaneous process driven by electrostatic interaction.

Voorn and Overbeek [15–21] showed quantitatively that the critical conditions for coacervation were met for a two component system consisting of a polyion salt and water when $\sigma^3 r \geq 0.53$. That is when either the molecular

charge density ($\sigma$) or the ratio of the molar volumes of solute to solvent ($r$), [$r$, is proportional to the polyion molecular weight] or both of these factors are sufficiently large. However complex coacervates have been formed when the above conditions are not met. It is possible to manipulate other factors, such as temperature to achieve coacervation [8, 22]. The assumption that the Huggins interaction parameter is negligible is therefore incorrect. Veis and Aranyi [22–25] developed a theory to explain a practical case of coacervation between two oppositely charged gelatins, where the Voorn–Overbeek theory did not apply and coacervates formed when the critical condition $\sigma^3 r \geq 0.53$ was not met. Coacervation occurred in this gelatin system on temperature reduction. Veis [23] modified the Voorn–Overbeek theory to include the Huggins interaction parameter, which increases significantly on temperature reduction. The Veis–Aranyi theory is limited to systems of low charge density [22]. Veis [23] considered that the nature of the electrostatic interaction was different than that calculated by Voorn and Overbeek from the Debye–Hückel theory [26, 27] since light scattering data indicated that aggregates were present in the equilibrium phase. These aggregates were thought to be ion-paired [22, 25]. Veis replaced the Debye–Hückel derived electrostatic term with a term which is a function of concentration and charge density and represents the electrostatic free energy change on the transfer of a polymer segment from the dilute to the concentrated phase [23].

The three basic assumptions of the Voorn–Overbeek theory do not hold for the Veis–Aranyi model. The molecules are not randomly distributed in both phases nor are the charges, since ion-paired aggregates are present in the dilute phase. The Huggins interaction parameter is not negligible, in fact it is the driving force for coacervation, since coacervation is induced on temperature reduction. Veis and Aranyi [22] proposed that complex coacervation of oppositely charged gelatins occurred in two steps. First the gelatins spontaneously aggregate by electrostatic interaction to form neutral aggregates of low configurational entropy, and then these aggregates coacervate to give phase separation. Coacervation is driven by the gain in configurational entropy resulting from the formation of a randomly mixed coacervate phase. In contrast, the Voorn–Overbeek theory [15–21] states that coacervation is a spontaneous process driven by electrostatic interaction. The differences between the Voorn–Overbeek and the Veis–Aranyi theories arise since each is attempting to explain very specific coacervation conditions. The Voorn–Overbeek theory was developed based on data obtained for the spontaneous formation of coacervation in the gelatin/acacia coacervate system, and the Veis–Aranyi theory was developed to account for coacervation between oppositely charged gelatins.

Two other complex coacervation theories have been developed: by Nakajima and Sato [11, 12], which is an adaptation of the Voorn–Overbeek theory, and by Tainaka [13, 14], which is an adaptation of the Veis–Aranyi theory. Nakajima and Sato agreed with the Voorn–Overbeek theory in that the charges should be treated as distributed uniformly in both phases, but included the Huggins interaction parameter and they altered the electrostatic term. The



Tainaka theory differs from that of Veis and Aranyi in that aggregates are considered to be present in both phases, which possibly neutral, are not formed by specific ion pairing. According to Tainaka, phase separation is driven by attractive forces among aggregates, which are stronger the higher the molecular weights and the greater the charge densities of the macromolecules. Unlike the Veis–Aranyi theory the Tainaka theory is not restricted to low charge density, however both the charge density and polyion molecular weight should fall within a critical range. Where the charge density or molecular weight values are higher than this range, a concentrated gel or precipitate will form since strong long range attractive forces will exist among the aggregates. At macromolecule charge densities and molecular weights below this range the dilute solution will be stabilized by short range repulsive forces and coacervation will not occur. The Tainaka theory is more general than the other theories and is applicable to both high and low charge density systems. However, it does not provide a full explanation of the coacervation process. Notably it does not explain suppression of coacervation at low ionic strength, which has been reported by Burgess and coworkers [8–10]. This theory does provide an adequate explanation of the complex coacervation process for a large number of systems.

## 17.5 Coacervation as a Method of Microencapsulation

Coacervation is a common method of microencapsulation [1, 8, 28–30]. The potential of encapsulation by coacervates was first noted by Bungenberg de Jong and Kaas [31] who observed that organic liquids suspended in the equilibrium phase of a coacervate system were taken up by the coacervate droplets. Bungenberg de Jong et al. [32] showed that solid particles could also be entrapped in coacervate systems. On phase separation by simple or complex methods tiny coacervate droplets are formed which coalesce and sediment to form a separate coacervate phase. If a core material, such as drug particles, is present in a polyion system prior to coacervation then the coacervate will deposit on and coalesce around these core particles (Fig. 17.3). Agitation of the coacervate system by stirring or other means can prevent coalescence and sedimentation of the coacervate droplets. The coacervate droplets can be crosslinked to form stable microcapsules by addition of a crosslinking agent, such as glutaraldehyde, or the use of heat [8, 28–30]. The first commercial application of this encapsulation technique was by the National Cash Register Company for the manufacture of carbonless copying paper [33]. The potential use of microencapsulation in the pharmaceutical industry has been considered since the 1960s [5, 34, 35]. Drug microencapsulation by coacervation has been reviewed by Madan [29] and by Nixon [30].

In the pharmaceutical industry, microencapsulation has many advantages, such as: to mask odor and taste; to convert oils into solid entities; to protect drugs against moisture, light, heat or oxidation; to delay volatilization; to separate incompatible materials during compounding of a medicine; to improve

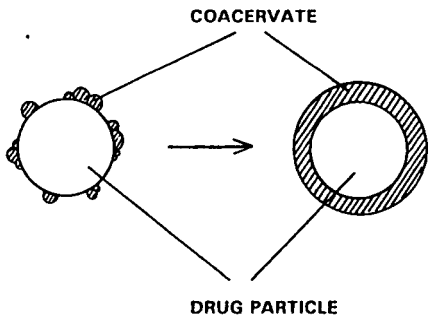


Fig. 17.3. Coacervate formation around a drug particle

the flow ability of powders; to handle toxic materials; to aid in the dispersion of water insoluble substances in aqueous media; and to produce sustained or controlled release medications [30, 36–38]. Microencapsulation has also been used medically for the encapsulation of live cells and vaccines [37, 39, 40]. The immobilization of mammalian cells within microcapsules has use in organ transplantation therapy [41]. The immobilized cells should be protected from the hosts' immune system and therefore should survive to deliver their product (such as insulin) in a bioresponsive manner for extended periods of time. In addition, the entrapped cells may be derived from a species other than the host. For example, bovine pancreatic islets could be used in the treatment of human diabetes.

Complex coacervate microencapsulation does not require any elaborate manufacturing equipment. The large number of variables which affect complex coacervation (pH, ionic strength, macromolecule concentration, macromolecule ratio and macromolecular weight [1, 7] affect microcapsule production, with the result that there are a large number of controllable parameters in this process. These parameters can be manipulated to produce microcapsules with specific properties. Complex coacervate systems which have been investigated as potential microencapsulation methods include: gelatin/acacia [42]; carbopol-gelatin [43]; pectin-gelatin [44]; gelatin/gelatin [8]; and sodium carboxymethylcellulose-gelatin [45]. Complex coacervate microcapsules have been formulated as suspensions or gels [46], and have been compounded within suppositories [47, 48] and tablets [49, 50]. Tablets containing microcapsules have been utilized to obtain a sustained release action. For example, sustained release sodium phenobarbitone tablets have been prepared containing simple coacervate, ethylcellulose, sodium phenobarbitone microcapsules [50]. Sustained release suppositories containing microencapsulated indomethacin have been successfully formulated [48]. These indomethacin suppositories showed a zero order release profile which did not decrease for 9 hours and approximately 100% of the indomethacine was released. The microcapsules were prepared by simple coacervation using ethylcellulose. Sustained release indomethacin suppositories have the following advantages: reduction of gastrointestinal irritation, avoidance of both disagreeable taste and first-pass effects; and reduction in the frequency of drug administration.



Hemoglobin-containing coacervate vesicles have been prepared by complex coacervation of gelatin with: acacia, pectin and dextran sulfate [51]. The hemoglobin is anchored into the vesicle walls by interaction of its polyanion binding site with the carbohydrates. Oxygen binding of the immobilized HbA is reversible and cooperative. Kinetic studies on $CO_2$ binding have shown that $CO_2$ uptake is similar to that of human erythrocytes. These microcapsules slowly dissolve in isotonic salt solutions unless they are stabilized by crosslinking with glutaraldehyde [51]. Hemoglobin incorporated into glutaraldehyde stabilized coacervates has functional properties similar to those of intraerythrocytic hemoglobin [51].

Although many successful coacervate microencapsulation systems have been prepared coacervate microcapsules have a number of limitations: they can be produced only at specific pH values; they require stabilization by use of crosslinking agents or heat; and the extent of crosslinking determines the retention of the encapsulant. The limitation of pH can be overcome on addition of water soluble nonionic polymers, such as polyethylene glycol [52, 53]. The addition of a small amount of these polymers, allows microencapsulation to occur over an expanded pH range (for example, the pH range for coacervation of gelatin and acacia can be extended from pH 2.6 to 5.5 [7] to pH 2 to 9 [52]. In addition these polymers induce simple coacervation [52], and this has been shown for macromolecules such as: gelatin, caboxymethylcellulose and ethylene-maleic anhydride copolymer. The pH range for simple coacervation is also expanded when these water soluble nonionic polymers are used. For example, the pH range for simple coacervation of gelatin was increased from the vicinity of the isoelectric point to the pH range 5.5 to 9.5 [52].

Glutaraldehyde and formaldehyde are commonly used as crosslinking agents for protein/polysaccharide complex coacervate systems such as gelatin and acacia. A condensation reaction occurs between the amino groups of the protein and the aldehydes. However there are potential toxicity problems when these chemicals are used in phramaceutical products. In addition, both the use of chemical crosslinking agents and the application of heat may be harmful to the encapsulant materials, such as thermo- and chemically labile drugs and live cells. Examples of drugs which may not be able to withstand these processing conditions are the many genetically engineered protein and polypeptide drugs.

Crosslinking of coacervates is necessary to stabilize the coacervate emulsion droplets and hence form microcapsules. A stable coacervate system formed without the use of chemical crosslinking agents or the application of heat would be potentially useful as a delivery system for protein and polypeptide drugs and other materials unable to withstand these crosslinking procedures. The relative stability of coacervate emulsion systems, as other emulsion systems, is dependent on: dispersed phase droplet size and viscosity, interfacial tension, interfacial charge and interfacial rigidity. Small droplet size, high viscosity, low interfacial tension, high interfacial charge and rigid interfacial films all contribute to more stable emulsion systems. The interfacial tension of aqueous coacervate systems is usually low and therefore this parameter cannot be effectively manipulated to

nhance stability. Values of the order of 1 $mNm^{-1}$ have been reported for gelatin/acacia complex coacervate systems [54]. Complex coacervates have a net zero charge since the charges carried by the macromolecules neutralize one another. Alteration of pH or ionic strength to change the charge carried by the coacervates will only affect the extent of coacervation and will not affect the net charge carried by the coacervates. The parameters which can be manipulated to affect coacervate stability are particle size, viscosity and interfacial rigidity.

Viscous coacervate systems include albumin and alginic acid [9] and albumin and acacia [55]. The albumin/alginic acid system tends to form complex precipitates in addition to complex coacervates except under very restricted conditions of pH, ionic strength and concentration and this limits the usefulness of this system. The albumin/acacia coacervate system also varies significantly with the conditions, particularly pH and ionic strength. pH and ionic strength control the charge carried by the macromolecules and therefore the electrostatic interactive forces. Charge also determines macromolecular shape, which affects the type of interaction and the amount of solvent entrapped within the associated macromolecules. The appearance of the albumin/acacia coacervate system changes markedly as the pH and ionic strength conditions are altered [55]. Interaction was observed over the pH range 2.0 to 6.0 and ionic strength range 0 to 100 mM [55]. The optimum conditions for maximum coacervate yield were pH 3.9 and ionic strength 10 mM. Under these conditions the system rapidly separates into a viscous coacervate phase and a dilute equilibrium phase. Close to the optimum conditions for maximum coacervate yield the system has a cloudy appearance and as the conditions are altered away from the optimum conditions the system becomes less cloudy and eventually clear.

The viscosity of the albumin/acacia system under the optimum conditions for maximum coacervate yield was too high to prepare microcapsules, the coacervate phase could not be emulsified into the equilibrium phase [55]. However it is considered that the highly dispersed coacervate systems which form at pH values close to the optimum conditions may be useful for microencapsulation. The coacervate yields obtained for these systems are high (between 80 and 89% w/v, [55]) and these highly dispersed systems appear to be relatively stable in the dispersed state compared to other coacervate systems. It was therefore decided to investigate this coacervate system as a potential microencapsulation system.

## 7.6 Materials and Methods

Bovine serum albumin (mol. wt. $6.7 \times 10^4$ [osmotic pressure], isoelectric pH 5.6), acacia (mol. wt. $2.4 \times 10^5$ [56]), Amberlite IR-120P (cation-exchanger), Amberlite IR-400 (anion-exchanger) and glutaraldehyde were obtained from Sigma Chemicals, USA. Gelatin B (Bloom No. 250, mol. wt. $4.6 \times 10^4$ [osmotic pressure], isoelectric pH 4.8), was obtained from Gelatin Products Ltd, U.K.



The isoelectric pH values of albumin and gelatin were measured by microelectrophoresis and by ion exchange [10]. Hydrochloric acid, sodium hydroxide, sodium chloride and other chemicals used were of analytical grade and obtained from Fisher Scientific, USA. The polyion solutions were prepared by dispersion in distilled water at $40 \pm 0.1$ °C. The polyions were allowed to hydrate completely; this took 30 min to 1 h. Following hydration the solutions were deionized by mixing for 30 min at $40 \pm 0.1$ °C with Amberlite resins IR-120P and IR-400 before use. This method is an adaptation of that of Janus et al. [57].

### *17.6.1 Albumin/Acacia Complex Coacervation and Microcapsule Production*

Albumin/acacia coacervates were prepared by mixing equal volumes of deionized polyion solutions with constant stirring (300 rpm) at $40 \pm 0.1$ °C for 1 h, at the appropriate pH and ionic strength conditions. The pH and ionic strength of the mixtures were adjusted using HCl, NaCl and NaOH solutions. The concentration of the polyion solutions was adjusted to give a final total polyion concentration of 2% w/v. The appearance of the caocervates was then examined visually.

Microcapsules were prepared by mixing 100 ml of each polyion solution prepared as above. The pH was adjusted to either pH 3.8 or 4.2, maintaining constant ionic strength at 10 mM using HCl, NaCl and NaOH solutions. The mixtures were treated in one of the following ways: (i) left to stand, no further treatment; (ii) stirred at 450, 600 or 900 rpm for 1 h using a Dyna mixer (Fisher Scientific); (iii) stirred at 450 rpm for 10 min using a Dyna mixer, followed by sonication for 10 min using a Sonifier, Branson Sonic Co., at a power setting of 5. Each batch was separated into two portions; one portion was left to stand, no further treatment; and 5 ml of crosslinking agent (16% glutaraldehyde solution) was added to the other portion, and mixing was continued for a further 5 min. When necessary, commassie blue dye was added to the acacia solution prior to mixing. The percentage of commassie brilliant blue present in the coacervate and equilibrium phases was determined by visible spectrophotometry at 550 nm.

### *17.6.2 Gelatin/Acacia Complex Coacervate Production*

Gelatin/acacia complex coacervates were produced as the albumin/acacia complex coacervates described above, with the exception that the pH was adjusted to pH 3.6 (the optimum pH for maximum coacervate yield [7]) maintaining constant ionic strength at 10 mM using NaCl, NaOH and HCl solutions.

### *17.6.3 Particle Size Stability Study*

The microcapsule samples prepared were stored for periods up to 42 days in constant temperature water baths at 5, 25 and $40 \pm 0.1$ °C. The particle size was

measured on days 1, 14 and 42. The mean diameter (dsn) and the size distribution of the microcapsules were determined using a HIAC/Royco particle sizer (Model 4100). During the HIAC/Royco particle size analysis the microcapsules were suspended in 0.9% w/v sodium chloride solution. Microcapsule samples were examined by optical and scanning electron microscopy. The stability of the microcapsules to dilution and change in pH and ionic strength was studied by altering the pH and ionic strength of the final microcapsule suspension to values within the pH range 3.0 to 9.0 and the ionic strength range 1 to 80 mM. Particle size analysis of the microcapsule suspensions was conducted as above using the HIAC/Royco particle sizer and the samples were examined microscopically.

## 17.7 Results

As reported previously albumin/acacia coacervate microcapsules could not be prepared at the optimum conditions for maximum coacervate yield, pH 3.9 and ionic strength 10 mM, due to the high viscosity of the coacervate phase [55]. Emulsification was attempted using high speed stirring and ultrasonic energy via an ultrasonic probe, however none of these methods was successful. At pH and ionic strength conditions close to the optimum coacervate yield conditions highly dispersed systems formed and these were investigated as potential microencapsulation systems. Two systems were selected for the preparation of coacervates: pH 3.8, ionic strength 10 mM; and pH 4.2, ionic strength 10 mM. Coacervates were prepared at different manufacturing conditions and particle size analysis was conducted to investigate the stability of these coacervates over a six week period. Each batch was divided in two and one portion was crosslinked with glutaraldehyde and the other portion was left untreated. The crosslinked sample was used as a control to determine any change in the particle size of the non-crosslinked coacervates with time. The batches were prepared under a range of different shear conditions, to determine the effect of shear rate on the particle diameter. Ultrasonication and different stirring speeds were utilized and some batches were left unstirred. The various batches were stored at 25 °C.

The coacervates were analyzed using a HIAC/Royco particle sizer and an optical microscope. All coacervate samples examined by microscopy were spherical and the mean particle diameters did not change over a six hour observation period.

Coalescence of coacervate droplets was not observed. For comparative purposes an identical study was conducted using gelatin/acacia coacervates which are known to coalesce readily. Accurate determination of the mean coacervate diameter of this system could not be made as coalescence occurred very rapidly. Over a period of 10 minutes all the gelatin/acacia coacervate droplets in the field of view had coalesced into one.



The mean particle diameters of different microcapsule batches are given in Tables 17.1 and 17.2 for coacervates prepared at pH 3.8 and 4.2, respectively. The mean particle diameters did not vary significantly. There was no apparent correlation between stirring rate and mean particle diameter. An increase in applied shearing force usually reduces the particle size of emulsion systems, thus smaller coacervate droplets would be expected with higher stirring speeds. The particle size data for the non-crosslinked and crosslinked coacervates were not significantly different, indicating that the non-crosslinked coacervate droplets are relatively stable.

**Table 17.1.** The effects of stirring rate and aging on the mean particle diameter of albumin/acacia non-crosslinked and crosslinked complex coacervates prepared at pH 3.8

| Stirring speed (rpm) | Mean particle diameter ($dsn \pm \delta g$ in μm) Day 1 | Day 14 | Day 42 |
|---|---|---|---|
| NS | 5.6 ± 1.4 | 5.6 ± 1.4 | 5.9 ± 1.3 |
| 450 | 5.7 ± 1.4 | 5.6 ± 1.4 | 5.5 ± 1.4 |
| 600 | 5.3 ± 1.5 | 5.5 ± 1.4 | 5.5 ± 1.4 |
| 900 | 5.4 ± 1.5 | 5.5 ± 1.3 | 5.6 ± 1.4 |
| sonicated | 5.5 ± 1.3 | 5.9 ± 1.2 | 5.9 ± 1.2 |
| NS* | 5.3 ± 1.5 | 5.0 ± 1.3 | 5.0 ± 1.4 |
| 450* | 5.7 ± 1.4 | 5.6 ± 1.4 | 5.5 ± 1.4 |
| 600* | 5.1 ± 1.4 | 5.3 ± 1.5 | 5.3 ± 1.4 |
| 900* | 5.4 ± 1.5 | 5.4 ± 1.5 | 5.6 ± 1.4 |
| sonicated* | 5.5 ± 1.3 | 5.7 ± 1.2 | 5.7 ± 1.3 |

* – crosslinked coacervates.
NS – Not stirred

**Table 17.2.** The effects of stirring rate and aging on the mean particle diameter of albumin/acacia non-crosslinked and crosslinked complex coacervates prepared at pH 4.2

| Stirring speed (rpm) | Mean particle diameter ($dsn \pm \delta g$ in μm) Day 1 | Day 14 | Day 42 |
|---|---|---|---|
| NS | 5.7 ± 1.2 | 6.0 ± 1.7 | 6.2 ± 1.5 |
| 450 | 5.7 ± 1.4 | 5.9 ± 1.6 | 5.8 ± 1.4 |
| 600 | 5.9 ± 1.5 | 6.1 ± 1.4 | 6.1 ± 1.4 |
| 900 | 5.1 ± 1.3 | 5.1 ± 1.3 | 5.2 ± 1.4 |
| sonicated | 5.5 ± 1.4 | 5.7 ± 1.4 | 5.5 ± 1.4 |
| NS* | 5.9 ± 1.6 | 6.1 ± 1.4 | 6.2 ± 1.5 |
| 450* | 5.8 ± 1.4 | 6.9 ± 1.5 | 6.4 ± 1.6 |
| 600* | 5.6 ± 1.5 | 5.9 ± 1.3 | 5.8 ± 1.4 |
| 900* | 5.2 ± 1.5 | 5.5 ± 1.4 | 5.6 ± 1.6 |
| sonicated* | 5.7 ± 1.4 | 5.8 ± 1.5 | 6.2 ± 1.6 |

* – crosslinked coacervates.
NS – Not stirred

The mean particle diameters did not change significantly on aging over a six week period (Tables 17.1 and 17.2). This data suggests that the non-crosslinked systems are extremely stable to coalescence. The slight variation in the mean particle diameters with time are probably a consequence of flocculation (as observed by optical microscopy). Non-crosslinked liquid coacervate systems generally coalesce rapidly and form a separate phase [1, 8, 58]. Coalescence was observed microscopically for the gelatin/acacia coacervate system studied, but not for the albumin/acacia system. The relative stability of the albumin/acacia system may be due to high viscosity, which results from strong interactions between albumin and acacia and between albumin and water. Albumin has a high percentage of polar side groups including: aspartic acid, glutamic acid and threonine; all of which contribute to strong interactions with water. It appears that on mixing albumin and acacia solutions, under specific conditions, complex coacervates form and these aggregate up to a critical size. This aggregation process is almost instantaneous. Once formed the aggregates are not susceptible to further aggregation, coalescence and/or Oswald ripening. Judging from the spherical shape of the coacervates they must be in the liquid state when they form. However once the critical size is reached the consistency of the droplets change and they gel. The semisolid nature of these coacervates is consistent with their unusual behavior, such as: no evidence of particle size growth with time; and particle size does not vary with applied shear. The gel consistency of the albumin/acacia coacervates was confirmed by observation of the coacervates using an optical microscope. The coacervates deform elastically on application of finger pressure via a coverslip. When this pressure is removed the coacervates regained their original shape. Figure 17.4 is a scanning electron micrograph of albumin/acacia coacervate microgels prepared at pH 4.2.

The albumin/acacia coacervates were stable to dilution with distilled water and were unaffected by change in pH and ionic strength over the ranges studied: pH 3.0 to 9.0 and ionic strength 1 to 80 mM. The mean particle diameters of the diluted coacervate systems were measured initially and after two weeks to

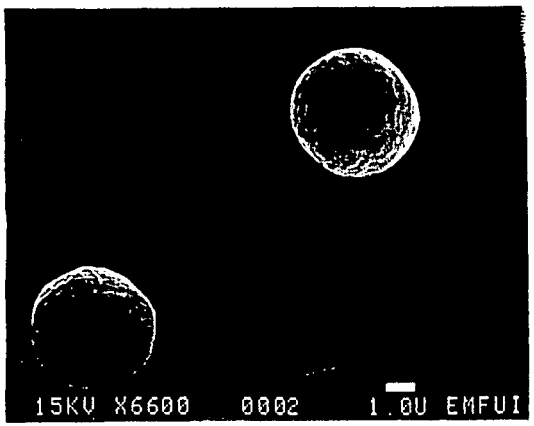


**Fig. 17.4.** Scanning electron micrograph of albumin/acacia coacervate microgels prepared at pH 4.2. The bar represents 1 μm



determine the stability of these systems. There was no significant difference in the mean particle diameters of the initial and the diluted samples. Liquid coacervate systems such as the gelatin/acacia system are unstable to change in pH and ionic strength and when these parameters are changed to values outside the coacervation range a one phase system forms.

Commassie brilliant blue, a water soluble dye was successfully encapsulated within the albumin/acacia coacervate system. Greater than 95% of the added dye was present within the microcapsules, as determined by spectrophotometric analysis. This indicates the usefulness of the albumin/acacia complex coacervate system as a method of microencapsulation.

The albumin/acacia system described appears to be in agreement with the Tainaka theory of complex coacervation. According to the Tainaka theory aggregates are present in both phases and coacervation is driven by attractive forces among the aggregates.

## 17.8 Conclusions

Albumin/acacia complex coacervates formed at pH and ionic strength values close to but not at the optimum conditions for maximum coacervate yield are stable with respect to: aging, temperature, pH, ionic strength and dilution (with and without glutaraldehyde crosslinking); as determined by particle size analysis. The relative stability of the coacervate microgels is apparently a consequence of the high viscosity of this system. This coacervate system appears to fit the Tainaka theory of complex coacervation. Albumin/acacia coacervate microgels, with average particle sizes of approximately 6 μm, have been prepared at pH and ionic strength values close to but not at the optimum conditions for maximum coacervate yield. Commassie brilliant blue dye was successfully encapsulated within these coacervates.

The albumin/acacia coacervate system is stable without the use of any crosslinking agents or the application of heat and therefore this system may be suitable for the encapsulation of drugs, biological agents and cells, which are unable to withstand the harsh processing conditions involved in other microencapsulation methods.

*Acknowledgments.* The author thanks Ms. H.K. Bhowra, Ms. T. Marek and Mr. O.N. Singh, University of Illinois at Chicago for technical assistance.

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# 18 Complexation of Proteins with Polyelectrolytes in a Salt-Free System and Biochemical Characteristics of the Resulting Complexes

E. Kokufuta

The present chapter deals with the complexation of proteins with polyelectrolytes in a salt-free system with regard to the following two aspects. The first is how an irregular distribution of ionizable groups attached along the length of inflexible protein molecules affects the stoichiometry of salt-linkage formation with oppositely charged flexible polyelectrolytes. And the second is to what extent biochemical ability is maintained in the resulting complexes. Three proteins, human serum albumin (HSA), human or bovine hemoglobin (Hb), and bovine trypsin (BT) were chosen as samples in view of the information available on their amino acid sequences, three-dimensional conformations, structures of biochemically active sites, etc. Poly(diallyldimethylammonium chloride) (PDDA) and potassium poly(vinyl alcohol sulfate) (KPVS), the polyion charges of which are independent of pH within the range 2 to 13, were employed as the polyelectrolytes. The stoichiometry of complexation was studied as a function of pH using a colloid titration method. The biochemical properties of the resultant stoichiometric complexes were examined in terms of the binding affinity of cyanide ions with the heme (ferri-protoporphyrin IX) in the ternary KPVS/Hb/PDDA complex, and also of the protease activity of the BT/KPVS complex. A model for stoichiometric complexes of proteins with polyelectrolytes is proposed on the basis of the results obtained. Also presented are the functional capabilities of the ternary KPVS/Hb/PDDA complex as a cyanide ion exchanger, and of the BT/KPVS complex as an immobilized enzyme.

## List of Symbols and Abbreviations

| | |
|---|---|
| ATEE | *N*α-acetyl-tyrosine ethyl ester |
| BAEE | *N*α-benzoyl-arginine ethyl ester |
| BANA | *N*α-benzoyl-arginine-*p*-nitroanilide |
| BT | bovine trypsin |
| BTNA | *N*α-benzoyl-tyrosine-*p*-nitroanilide |
| *C* | molar concentration |
| *D* | optical density |
| Hb | hemoglobin |
| HbCN | cyanide methemoglobin |
| HbOH | alkaline methemoglobin |
| $Hb^+OH_2$ | acidic methemoglobin |
| HSA | human serum albumin |
| *K* | equilibrium constant |
| KPVS | potassium poly(vinyl alcohol sulfate) |
| LEE | leucine ethyl ester |